**NMR Metabolomics Methods for Barth Syndrome Study**

Aliquots of plasma samples (125 µL) were transferred into pre-labeled 2.0 mL low-bind Eppendorf tubes for experimental samples. For all samples 125 µL of 0.9% Saline in D2O (containing 2 mM Formate (chemical shift indicator) and 0.2% NaN3 (to prevent bacterial growth)) was added to each tube and were vortexed for 2 min at 5000 rpm. The samples were then centrifuged at 16,000 rcf for 2 min. Total pools of the samples were prepared (by using 12 µL of each study sample) to serve as QC samples, and 125 µL aliquots of QC samples were processed in the same way as described above for study samples. A 200 µL aliquot of the samples were then transferred into 3 mm NMR tubes (Bruker-Biospin, Switzerland), which were kept in a cooler with freeze packs until data acquisition.

1H NMR spectra of plasma samples were acquired on a Bruker Avance III 950 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using a 5 mm cryogenically cooled ATMA inverse probe and ambient temperature of 25℃. A cpmgpr1d pulse sequences used for data acquisition. For each sample 128 transients were collected into 64k data points using a spectral width of 12.03 ppm, 2 s relaxation delay, 400 µs fixed echo time, loop for T2 filter (l4)=80, and an acquisition time of 2.866 s per FID. The water resonance was suppressed using resonance irradiation during the relaxation delay. Spectra were zero filled, and Fourier transformed after exponential multiplication with line broadening factor of 0.5. Phase and baseline of the spectra were manually corrected for each spectrum. Spectra were referenced internally to the formate signal. The quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers. Spectra were assessed for missing data and underwent quality checks.

NMR spectra were pre-processed using ACD 1D NMR Processor 12.0 (ACD Labs, Toronto, CA) for metabolomics analysis. NMR bins (0.70 – 7.75 ppm) were made after excluding water (4.15 – 5.15 ppm) using intelligent binning width of 0.04 ppm and 50% looseness factor. Integrals of each of the bins were normalized to the total integral of each of the spectrum.

There were four samples noted to be potentially EDTA plasma, rather than heparinized. These samples are noted in the binned data spreadsheet and were excluded from the analysis.